



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US97/11508 <b>(22) International Filing Date:</b> 30 June 1997 (30.06.97) <b>(30) Priority Data:</b> 60/022,180 19 July 1996 (19.07.96) US 08/742,805 1 November 1996 (01.11.96) US 08/812,208 6 March 1997 (06.03.97) US <b>(71) Applicant:</b> IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC. [US/US]; Office of Intellectual Property & Technology Transfer, 310 Lab of Mechanics, Ames, IA 50011-2131 (US). <b>(72) Inventors:</b> ROTHCHILD, Max, F.; Iowa State University Research Foundation, Inc., 310 Lab of Mechanics, Ames, IA 50011-2131 (US). VINCENT, Amy, L.; Iowa State University Research Foundation, Inc., 310 Lab of Mechanics, Ames, IA 50011-2131 (US). TUGGLE, Christopher, K.; Iowa State University Research Foundation, Inc., 310 Lab of Mechanics, Ames, IA 50011-2131 (US). <b>(74) Agent:</b> NEBEL, Heidi, Sease; Zarley, McKee, Thomte, Voorhees & Sease, Suite 3200, 801 Grand Avenue, Des Moines, IA 50309-2721 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PROLACTIN RECEPTOR GENE AS A GENETIC MARKER FOR INCREASED LITTER SIZE IN PIGS  <p style="text-align: center;"> <u>AAGTCAACAA AGATGGAGCA CTGGCGTTGC TCCCAAAACA GCAGGAGAAC</u>        GCGGACCGGC CGGAGAAGGC TGGCGCCCCT GAAACCAGCA AGGAATACG        CCCAGGTGTC CCGGGTGATG GATAACCACA TCCTGGTGTT AGTGCAGGAT        CCGCGAGCTC GAAACGTGGC TCCGTTTGAA GAACCAACCA AGGAGACCCC        GCCATCCCGG CCGCAGAATC CAGCTGCGAA AGACCTGGCC G/AGCTTCACCA        CGGCCCCGGG CCACTGCAGA CACCCGCTGG GTGGGCTGGA TTACCTCGAT        CCCGCAGGCT TTATGCACTC CTTTCAGTGA GAGCTTGGTT CATGGGATGA        TGGGTTACAA GGTGGGGTTT TTTTCAGGTC GCACTACGTG AAATGCACTC        TACCAGAGAA AGCTCGAAAA TGGGGTTAGA ATGACACTAC CCAGACTCAC        AGTTCACTCC TCTTCATGCT <u>CCATTTTCAA CCACCTGCCTCTT</u> </p> <p style="text-align: center;">G/A=G or A at polymorphic site</p>		
<b>(57) Abstract</b>  Disclosed herein are genetic markers for pig litter size, methods for identifying such markers, and methods of screening pigs to determine those more likely to produce larger litters and preferably selecting those pigs for future breeding purposes. The markers are based upon the presence or absence of certain polymorphisms in the pig prolactin receptor gene coding region.		

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TITLE: PROLACTIN RECEPTOR GENE AS A GENETIC MARKER  
FOR INCREASED LITTER SIZE IN PIGS

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FIELD OF THE INVENTION

This invention relates generally to the detection of genetic differences for reproductive efficiency among pigs and particularly use of a genetic marker, the prolactin receptor gene, which is indicative of the heritable trait of increased litter size.

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BACKGROUND OF THE INVENTION

Reproductive efficiency, which can be defined as the number of pigs produced per breeding female, is the major limiting factor in the efficient production of pork. The number of pigs born alive in the United States averages approximately 9.5 pigs per litter. Heritability for litter size is low (10%-15%), and standard genetic methods of selecting breeding females on the basis of past litter size have not been effective. Therefore, there is a need for an approach that deals with selection for reproduction at the cellular or DNA level.

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Chinese breeds are known for reaching puberty at an early age and for their large litter size. American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of breeds, thereby improving the efficiency of U.S. pork production. These efforts would be greatly assisted by the discovery of genes or genetic markers that are associated with increased litter size in pigs.

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RFLP analysis has been used by several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989), incorporated herein by reference, discloses the use of RFLP techniques to show genetic variability between two pig breeds. Polymorphism was demonstrated for swine leukocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting of Midwestern Section of the American Society of Animal Science, March 26-28, 1990, incorporated herein by reference, reports on the polymorphism of swine major histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al. Animal Genetics, 26:79-91 (1989), incorporated herein by reference, reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the

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results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further state that the use of SLA Class I restriction fragments, as genetic markers, may have potential in the future for improving pig growth performance.

5 Further, United States Patent 5,550,024 to Rothschild et. discloses a polymorphism in the pig estrogen receptor gene which is associated with larger litter size, the disclosure of which is incorporated herein by reference.

Another pig hormone related to reproductive success is Prolactin. Prolactin (PRL) is an anterior pituitary peptide hormone involved in many  
10 different endocrine activities, but is essential for reproductive success. One of its best characterized functions is regulating milk production in adult mammals. PRL is required for the stimulation of lactogenesis, or synthesis of milk proteins. This action is mediated by its receptor (PRLR). PRLR belongs to the cytokine/GHR/PRLR superfamily. When activated by PRL, PRLR  
15 begins a signal transduction pathway, which ultimately activates transcription of genes such as  $\beta$ -casein and  $\alpha$ -lactalbumin. When activated by PRL, PRLR begins a signal transduction pathway thought to involve the tyrosine kinase Jak2. Mutations in the carboxy-terminal end of the protein which change a specific phosphotyrosine residue prevents the receptor from activating Jak2  
20 and ultimately interferes with the activation of transcription of the  $\beta$ -casein gene (Lebrun). Long and short forms of the receptor protein, as well as various transcript sizes have been characterized in the rat, mouse, rabbit, and human. (Boutin, Edery, Lesueur). However, it has been demonstrated that the short form is not capable of activating transcription of the milk protein genes. The  
25 mRNAs seen thus far originate from the same primary transcript and are due to alternative splicing, specifically in the untranslated regions in the rabbit and human. Recently, PRL has also been shown to stimulate the production of progesterone, required for maintenance of pregnancy, in large porcine luteal cells in vitro. PRLR is thought to mediate the effects of growth hormone (bST) injections on higher milk yield in cattle, and thus may be important in varying  
30 milk yield in pigs. In humans and mice, the growth hormone receptor (GHR) and PRLR map close together (Arden et al., 1990; Barker et al., 1992), making it likely that these two genes are linked in pigs. GHR has been mapped in pigs to chromosome 16, while PRLR is unmapped and no genetic variability has  
35 been reported for PRLR.

The present invention provides a genetic marker, based upon the discovery of polymorphisms in the prolactin receptor gene, which relates to

increased average litter size in pigs. This will permit genetic typing of pigs for their prolactin receptor genes and for determination of the relationship of specific RFLPs to increased litter size. It will also permit the identification of individual males and females that carry the gene for larger litters. In the case of females it would permit that a female would be expected to produce a litter size larger than the average for their breed, or in the case of males for their female offspring to have larger litters than the breed average. Thus, the markers will be selection tools in breeding programs to develop lines and breeds that produce litters containing a larger number of offspring.

### SUMMARY OF THE INVENTION

It is an object of the invention to provide a method of screening pigs to determine those more likely to produce larger litters.

Another object of the invention is to provide a method for identifying genetic markers for pig litter size.

A further object of the invention is to provide genetic markers for pig litter size.

Yet another object of the invention is to provide a kit for evaluating a sample of pig DNA for specific genetic markers of litter size.

Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of the instrumentality's and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for screening pigs to determine those more likely to produce a larger litter when bred or to select against pigs which have alleles indicating smaller litter sizes. As used herein "larger litters" means a significant increase in litter size above the mean of a given population. Thus, the present invention provides a method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps 1) obtaining a sample of genomic DNA from a pig; and 2) analyzing the genomic DNA obtained in 1) to determine which prolactin receptor allele(s) is/are present. Briefly, a sample of genetic material is obtained from a pig, and the sample is analyzed to determine the presence

or absence of a polymorphism in the coding region of the prolactin receptor gene that is correlated with increased litter size.

In a preferred embodiment the polymorphism is a restriction fragment length polymorphism and the assay comprises identifying the pig prolactin receptor gene from isolated pig genetic material; exposing the gene to a restriction enzyme that yields restriction fragments of the gene of varying length; separating the restriction fragments to form a restriction pattern, such as by electrophoresis or HPLC separation; and comparing the resulting restriction fragment pattern from a pig prolactin receptor gene that is either known to have or not to have the desired marker. If a pig tests positive for the marker, such pig can be considered for inclusion in the breeding program. If the pig does not test positive for the marker genotype the pig can be culled from the group and otherwise used.

In a most preferred embodiment the gene is isolated by the use of primers and DNA polymerase to amplify a specific region of the gene which contains the polymorphism. Next the amplified region is digested with a restriction enzyme and fragments are again separated. Visualization of the RFLP pattern is accomplished by simple staining of the fragments, or by labeling either the primers or the nucleoside triphosphates used in amplification.

In another embodiment, the invention comprises a method for identifying a genetic marker for pig litter size in a particular population. Male and female pigs of the same breed or breed cross or similar genetic lineage are bred, and the number of offspring produced by each female pig is determined. A polymorphism in the prolactin receptor gene of each pig is identified and associated with the number of offspring. Preferably, RFLP analysis is used to determine the polymorphism, and most preferably, the DNA is digested with the restriction endonuclease AluI.

It is also possible to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene (e.g. the prolactin receptor gene discussed herein), which have previously been shown to be associated with a particular trait. Thus, in the present situation, taking the prolactin receptor gene, it would be possible, at least in the short term, to select for pigs likely to produce larger litters, or alternatively against pigs likely to produce smaller litters, indirectly, by selecting for certain alleles of a prolactin receptor associated marker through the selection of specific alleles of alternative chromosome 16 markers.

Examples of such markers known to be linked to prolactin receptor on porcine chromosome 16 includes SW1305, S0077, S0006, SW2411, SW1035 and S0111, which markers are all microsatellites and Growth Hormone Receptor (GHR).

The invention further comprises a kit for evaluating a sample of pig DNA for the presence in pig genetic material of a desired genetic marker located in the pig prolactin receptor gene indicative of the inheritable trait of large litter size. At a minimum, the kit is a container with one or more reagents that identify a polymorphism in the pig prolactin receptor gene. Preferably, the reagent is a set of oligonucleotide primers capable of amplifying a fragment of the pig prolactin receptor gene that contains the polymorphism. Preferably, the kit further contains a restriction enzyme that cleaves the pig prolactin receptor gene in at least one place. In a most preferred embodiment the restriction enzyme is AluI or one which cuts at the same recognition site.

The accompanying figures, which are incorporated herein and which constitute a part of this specification, illustrates one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

## DESCRIPTION OF THE FIGURES

Figure 1 depicts the sequence of the 3' coding and untranslated region of the pig prolactin receptor gene (SEQ ID NO:3). The pig PCR fragment produced from the rabbit/human primers was purified using Amicon Microcon Filters as directed (Amicon, Inc.). Sequencing was done by the Iowa State University DNA Sequencing and Synthesis Facility. Region in italics represents ambiguity in sequence and may be *ccaaaactac* (SEQ ID NO:3) → Pig PCR primers. — Rabbit/human sequence.

Figure 2 depicts the Polymorphic pattern of AluI digested PCR product. The forward primer 5'-CCC AAA ACA GCA GGA GAA CG-3' and the reverse primer 5'-GGC AAG TGG TTG AAA ATG GA-3' were used in the following PCR conditions: 93°C for 3 minutes, and 35 cycles of 93°C 30 seconds, 60°C 1 minute, 70°C 1 minute, and a final 72°C 3 minutes. The Taq Polymerase was added last while samples were held at 80°C. PCR products were cut with Alu I (New England Biolabs) and separated on a 6% NuSieve (FMC) agarose gel at 120 volts for 4 hours at room temperature. Gels were stained with ethidium bromide. Lane 1 is 1-kb ladder, lanes 2-4 are the three different genotypes.

Figure 3 depicts the position of PRLR in pig chr 16. Multiple point linkage was done using CriMap to produce a sex-averaged best-map with a LOD score of 3 or greater being considered as significant.

Figure 4 is a diagram of the fragments obtained from the PCR test using  
5 PCR primers SEQ ID NOS: 1 and 2.

### DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently referred  
embodiments of the invention, which together with the following examples,  
10 serve to explain the principles of the invention.

The invention relates to genetic markers for litter size in pigs. It provides a method of screening pigs to determine those more likely to produce a larger litter when bred by identifying the presence or absence of a polymorphism in the prolactin receptor gene that is correlated with increased  
15 litter size. As used herein, the term "increased litter size" means a biologically significant increase in litter size above the mean of a given population.

Thus, the invention relates to genetic markers and methods of identifying those markers in a pig of a particular breed, strain, population, or group, whereby the female pig is more likely to produce a litter that is  
20 significantly increased in size (number) above the mean litter size for that particular breed, strain, population, or group. Any method of identifying the presence or absence of this marker may be used, including for example single-strand conformation polymorphism (SSCP) analysis, RFLP analysis, heteroduplex analysis, denaturing gradient gel electrophoresis, and  
25 temperature gradient electrophoresis, ligase chain reaction or even direct sequencing of the prolactin receptor gene and examination for the AluI recognition pattern, in the 3' translated region.

Other possible techniques include non-gel systems such as TaqMan™ (Perkin Elmer). In this system oligonucleotide PCR primers are designed that  
30 flank the mutation in question and allow PCR amplification of the region. A third oligonucleotide probe is then designed to hybridize to the region containing the base subject to change between different alleles of the gene. This probe is labeled with fluorescent dyes at both the 5' and 3' ends. These dyes are chosen such that while in this proximity to each other the  
35 fluorescence of one of them is quenched by the other and cannot be detected. Extension by Taq DNA polymerase from the PCR primer positioned 5' on the template relative to the probe leads to the cleavage of the dye attached to the



5' end of the annealed probe through the 5' nuclease activity of the Taq DNA polymerase. This removes the quenching effect allowing detection of the fluorescence from the dye at the 3' end of the probe. The discrimination between different DNA sequences arises through the fact that if the hybridization of the probe to the template molecule is not complete, i.e. there is a mismatch of some form, the cleavage of the dye does not take place. Thus only if the nucleotide sequence of the oligonucleotide probe is completely complimentary to the template molecule to which it is bound will quenching be removed. A reaction mix can contain two different probe sequences each designed against different alleles that might be present thus allowing the detection of both alleles in one reaction.

The use of RFLPs is a preferred method of detecting the polymorphism. However, since the use of RFLP analysis depends ultimately on polymorphisms and DNA restriction sites along the nucleic acid molecule, other methods of detecting the polymorphism can also be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

RFLP analysis in general is a technique well-known to those skilled in the art. See, for example, U.S. Patents 4,582,788 issued April 15, 1986 to Erlich and 4,666,828 issued May 19, 1987 to Gusella, 4,772,549 issued September 20, 1988 to Frossard, and 4,861,708 issued August 29, 1989 to Frossard, all of which are incorporated herein by reference. Broadly speaking, the technique involves obtaining the DNA to be studied, digesting the DNA with restriction endonucleases, separating the resulting fragments, and detecting the fragments of various genes.

In the present invention, a sample of genetic material is obtained from a pig. Samples can be obtained from blood, tissue, semen, etc. Generally, peripheral blood cells are used as the source, and the genetic material is DNA. A sufficient amount of cells are obtained to provide a sufficient amount of DNA for analysis. This amount will be known or readily determinable by those skilled in the art. The DNA is isolated from the blood cells by techniques known to those skilled in the art.

Next the region containing the polymorphism is amplified by the use of primers and standard techniques, such as the polymerase chain reaction. This technique is described in U.S. Patents 4,683,195, issued July 28, 1987 to Mullis et al., 4,683,202, issued July 28, 1987 to Mullis, 4,800,159 issued January 24, 1989 to Mullis, et al., 4,889,818 issued December 26, 1989 to

Gelfand, et al., and 4,902,624, issued February 20, 1990 to Clumbus, et al., all of which are incorporated herein by reference. The selection of primers is discussed in the references mentioned and incorporated herein. The primers should amplify the 3' coding region and untranslated region of the pig

5 prolactin receptor gene as is demonstrated in Figure 2. Other such primers are designable by those of skill in the art combined with the teachings herein.

The isolated DNA is then digested with a restriction endonuclease that cleaves or cuts DNA hydrolytically at a specific nucleotide sequence, called a restriction site. Such endonucleases, also called restriction enzymes, are well-  
10 known to those skilled in the art. For the present invention, one should be chosen that cleaves the pig prolactin receptor gene coding region in at least one place, producing at least two fragments of the gene. A determination is made as to whether or not any such fragments are polymorphic and if any polymorphism (RFLP) is associated with litter size by techniques known in the  
15 art in conjunction with the teachings contained herein. Preferably, the restriction endonuclease is AluI. AluI cuts double stranded DNA at the sequence 5'-AGCT-3'. The amount of such enzyme to be added to the sample containing the pig DNA and the other appropriate conditions for treating the sample will be readily determinable to persons skilled in the art, given the  
20 teachings contained herein.

The restriction fragments are then analyzed by known techniques that generally involve either the separation of the fragments and visualization by staining or subsequent blotting and hybridization to obtain a particular pattern or the determination of different sizes of the fragments. The latter  
25 permits the identification of one or more fragments (markers) for increased litter size. The preferred separation technique is gel electrophoresis.

In this technique, the digested fragments are separated in a supporting medium by size under the influence of an applied electric field. Gel sheets or slabs, such as agarose or acrylamide, are typically used as the supporting  
30 medium. The sample, which contains the restriction fragments, is added to one end of the gel. One or more size markers are run on the same gel as controls to permit an estimation of the size of the restriction fragments. This procedure generally permits a degree of resolution that separates fragments that differ in size from one another by as little as 1 or 2 base pairs.

35 In alternative embodiments, the fragments are denatured and transferred physically from the gel onto a solid support, preferably a nylon

membrane, by contacting the gel with the filter in the presence of appropriate reagents and under appropriate conditions that promote the transfer of the DNA. Such reagents and conditions are well-known to those skilled in the art. Thus, the relative positions of the DNA fragments resulting from the separation procedure are maintained.

The next step involves the detection of the various categories of sizes of the fragments or, alternatively, the detection of a fragment of a particular size. The latter may be of particular interest because it is a genetic marker associated with increased litter size. This is preferably accomplished via staining of the fragments with ethidium bromide or the like.

An alternative technique is the use of a hybridization probe. Such a probe is an oligonucleotide or polynucleotide that is sufficiently complementary or homologous to the fragments to hybridize with them, forming probe-fragment complexes. Preferably, the probe is a cDNA probe. The oligonucleotide or polynucleotide is labeled with a detectable entity. This permits the detection of the restriction fragments, to which the probes are hybridized. The probes are labeled by standard labeling techniques, such as with a radiolabel, enzyme label, fluorescent label, biotin-avidin label, and the like. See U.S. patent nos. 4,711,955 issued December 8, 1987 to Ward et al. and 4,868,103 issued September 19, 1989 to Stavrianopoulos et al., both of which are incorporated herein by reference.

The probes are contacted with the nylon membrane that contains the restriction fragments for a sufficient period of time and under appropriate hybridizing conditions for the probes to hybridize to the fragments. The filter is then preferably washed to remove unbound probes and other unwanted materials.

The probe-fragment complexes, which are bound to the filter, are then detected by known techniques. For example, if the probe has been radioactively labeled ( $^{32}\text{P}$ ), detection involves contacting the nylon membrane paper with a piece of radiosensitive film. Following an appropriate exposure period, the fragments of interest, including control fragments, are visualized.

The detection step provides a pattern, resulting from the separation of the fragments by size. Comparison of these fragments with control fragments of known size that have also been run on the same gel permits the estimation of the size of the various groups of fragments. The various polymorphisms in the pig prolactin receptor gene are then determined by comparison of the patterns produced by similar analysis of DNA from a number of different pigs.

For some of the individual pigs, the patterns will differ from the usual pattern produced by most of the other pigs. This will be due to one or more restriction fragment length polymorphisms, i.e., restriction fragments of a different length produced by the endonuclease that cuts the pig prolactin receptor gene.

5 This indicates different base pair sequences in such pigs.

Once a particular RFLP has been identified, i.e., a restriction fragment of a particular length, a probe to this fragment may be constructed by the use of known techniques. This permits alternative and faster formats for detecting such polymorphism. For example, once the DNA is digested, a sandwich  
10 hybridization format can be used. Such an assay is disclosed in US. Patents 4,486,539 issued December 4, 1984 to Ranki, et al., and 4,563,419 issued January 7, 1986 to Ranki, et al., both of which are incorporated herein by reference. The sample is brought into contact with a capture probe that is immobilized on a solid carrier. The probe binds the fragment. The carrier is  
15 then washed, and a labeled detection probe is added. After additional washing, the detection probe is detected, thereby demonstrating the presence of the desired fragment.

In yet another embodiment, once the RFLP pattern has been determined or a particular polymorphic fragment has been determined, it is  
20 compared to a second, known RFLP pattern or fragment that is correlated with increased litter size. This second pattern or fragment has also been determined from the pig prolactin receptor gene, using the same restriction endonuclease as the first and the same probe or an equivalent thereof under the same conditions.

25 In an alternative embodiment of the invention, the restriction fragments can be detected by solution hybridization. In this technique, the fragments are first hybridized with the probe and then separated. The separated probe-fragment complexes are then detected as discussed above. Generally, such complexes are detected on the gel without transfer to filter paper.

30 In a most preferred embodiment the polymorphism is detected by PCR amplification without any probe. This procedure is known to those of skill in the art and is disclosed in U.S. Patents 4,795,699 entitled "DNA Polymerase" and U.S. Patent 4,965,188 "Process for Amplifying, Detecting, and/or Cloning Nucleic Sequences Using a Thermostable Enzyme" both of which are  
35 incorporated herein by reference.

For this procedure primers are constructed to amplify the region in which the polymorphism lies. Accordingly primers which are preferably 4-30

bases are designed based upon the sequence surrounding the polymorphism including a forward 5', primer and a reverse or anti-sense primer 3' of the polymorphism. The primers need not be the exact complement, and substantially equivalent sequences are also acceptable. A DNA polymerase is then added such as Taq polymerase (many such polymerases are known and commercially available) in the presence of the four nucleoside triphosphates and often a buffering agent. Detection is facilitated by simple staining, such as with ethidium bromide, of separated products to detect for predicted sizes based upon the length of the region amplified. Reaction times, reagents, and design of primers are all known to those of skill in the art and are discussed in the patents incorporated herein by reference. Further PCR amplification may be used in combination with Single Strand Confirmation Polymorphism (SSCP). See Detection of Polymorphism, of Human DNA by Gel Electrophoresis as Single-Strand Conformation Polymorphisms, Orita et al, PNAS 86(8) Apr. 1989 (2766-70); and Lessa et al. Mol Ecol 2(2) p. 119-29 Apr 1993 "Screening Techniques for Detecting Allelic variation in DNA Sequences" which are incorporated by reference.

Although the above methods are described in terms of the use of a single restriction enzyme and a single set of primers, the methods are not so limited. One or more additional restriction enzymes and/or probes and/or primers can be used, if desired. Additional enzymes, constructed probes and primers can be determined through routine experimentation.

Genetic markers for pig litter size are determined as follows. Male and female pigs of the same breed or breed cross or derived from similar genetic lineages are mated. The number of offspring produced by each female pig is determined. RFLP analysis of the parental DNA is conducted as discussed above in order to determine polymorphisms in the prolactin receptor gene of each pig. The polymorphisms are associated with the number of offspring. At least 20 and preferably at least 40 female pigs are used in making these determinations. The number of times each female produces a litter (i.e., the parity) is at least 1 time. Preferably, the cycle of breeding and giving birth is repeated at least 2 times and most preferably 3 times.

When this analysis is conducted and the polymorphism is determined by PCR RFLP analysis using the restriction endonuclease AluI, and amplification primers may be designed using analogous human or rabbit known prolactin sequences due to the high homology in the region surrounding the polymorphism, or may be designed using known pig prolactin gene sequence

data as exemplified in Figure 1 or even designed from sequences obtained from linkage data from closely surrounding genes. According to the invention a set of primers have been selected which amplify a 457 base pair fragment (forward primer 5'-CCC AAA ACA GCA GGA GAA CG-3' (SEQ ID NO:1) and the  
5 reverse primer 5'- GGC AAG TGG TTG AAA ATG GA-3' (SEQ ID NO:2)) after restriction polymorphic fragments of approximately 124, 110, 79, 77, and 67 base pairs are generated. The polymorphic site is located in the 110 base pair fragment. When the polymorphic cut site is present a 90 base pair fragment is produced. The polymorphic fragments were shown to be alleles, and each was  
10 shown to be associated with increased litter size for various breeds. Thus a pig which is heterozygous for the AluI fragment will exhibit a pattern of 124,110,90,79,77 and 67. A homozygote for the polymorphic cut site will exhibit a pattern of 124, 90, 79,77,67, while the other homozygote exhibits a pattern of 124,110, 79,77,67. The genotype associated with larger litter size  
15 alternates for different breeds. This outcome is similar to the situation disclosed in U.S. patent 5,374,523 entitled "Allelic variants of Bovine Somatotropin gene: Genetic marker for Superior Milk Production in Bovine" where the inventor found an allelic polymorphism in the somatotropin gene and one allelic form was beneficial for jersey cows and the alternate form was  
20 beneficial for Holstein cows.

The reagents suitable for applying the methods of the invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. At a minimum, the kit contains a reagent that identifies a polymorphism in the pig prolactin receptor gene that is  
25 associated with an increased litter size. Preferably, the reagent is a PCR set (a set of primers, DNA polymerase and 4 nucleoside triphosphates) that hybridize with the pig prolactin receptor gene or a fragment thereof. Preferably, the PCR set and a restriction enzyme that cleaves the pig prolactin receptor gene in at least one place are included in the kit. In a particularly preferred  
30 embodiment of the invention, the primer is SEQ ID NO:1 or SEQ ID NO:2 and the restriction enzyme is AluI. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization etc. may also be included, if desired.

35 The methods and materials of the invention may also be used more generally to evaluate pig DNA, genetically type individual pigs, and detect genetic differences in pigs. In particular, a sample of pig genomic DNA may be

evaluated by reference to one or more controls to determine if a polymorphism in the prolactin receptor gene is present. Preferably, RFLP analysis is performed with respect to the pig prolactin receptor gene, and the results are compared with a control. The control is the result of a RFLP analysis of the pig prolactin receptor gene of a different pig where the polymorphism of the pig prolactin receptor gene is known. Similarly, the prolactin receptor genotype of a pig may be determined by obtaining a sample of its genomic DNA, conducting RFLP analysis of the prolactin receptor gene in the DNA, and comparing the results with a control. Again, the control is the result of RFLP analysis of the prolactin receptor gene of a different pig. The results genetically type the pig by specifying the polymorphism in its prolactin receptor genes. Finally, genetic differences among pigs can be detected by obtaining samples of the genomic DNA from at least two pigs, identifying the presence or absence of a polymorphism in the prolactin receptor gene, and comparing the results.

These assays are useful for identifying the genetic markers relating to litter size, as discussed above, for identifying other polymorphisms in the prolactin receptor gene that may be correlated with other characteristics, and for the general scientific analysis of pig genotypes and phenotypes.

The genetic markers, methods, and kits of the invention are also useful in a breeding program to improve litter size in a breed, line, or population of pigs. Continuous selection and breeding of sows that are at least heterozygous and preferably homozygous for a polymorphism associated with increased litter size would lead to a breed, line, or population having higher numbers of offspring in each litter of the females of this breed or line. Thus, the markers are selection tools.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. The examples of the products and processes of the present invention appear in the following examples.

### EXAMPLE 1

Due to their high sequence homology and similarity in transcript processing, human (Boutin et al. 1989) and rabbit (Edery et al. 1989) cDNA sequences encoding the prolactin receptor were used to design degenerate primers overlapping the 3' coding and untranslated region. The primers amplified a fragment of approximately 500 base pairs in pig genomic DNA samples and human control. The forward primer 5'-TCA CAA GGT CAA C/TAA AGA TG-3' (SEQ ID NO:4) and the reverse primer 5'-TGG/A AGA AAG/A AGG CAA G/ATG GT-3' (SEQ ID NO:5) were used in the following PCR conditions: 93°C for 3 minutes, 6 cycles of 93°C 30 seconds, 47°C 2 minutes, 72°C 3 minutes, 36 cycles of 93°C 30 seconds, 53°C 2 minutes, 72°C 5 minutes, and a final 72°C 5 minutes. The Taq polymerase was added last while samples were held at 80°C.

Fragments from two animals were purified and sequenced in forward and reverse directions. The pig sequence from the coding region was translated to amino acids and compared with known sequences. A database search reported the rabbit and human PRLR sequences as the two best matches, with 82% and 74% positives respectively. From the pig DNA sequence, primers (forward primer 5'-CCC AAA ACA GCA GGA GAA CG-3' (SEQ ID NO:1) and the reverse primer 5'-GGC AAG TGG TTG AAA ATG GA-3' (SEQ ID NO:2)) were designed to amplify a 457 base pair fragment (Figure 1). The restriction endonucleases TaqI, Sau3a, PvuII, MspI, and AluI, were used to digest the amplified product and a polymorphism was found with AluI in the coding region of the gene. Resolution of the bands was obtained using agarose gel electrophoresis (Figure 2). The fragment sizes of the PCR-RFLP were approximately 124, 110, 79, 77, and 67 base pairs with the polymorphic site being located in the 110 base pair fragment. When the polymorphic cut site was present a 90 base pair fragment was produced. See Figure 4 for the fragment patterns produced. The PiGMap reference families (Archibald et al. 1995) were genotyped, with all available families being informative. The genotypes were analyzed for 2-point linkage using CriMap software (Green et al. 1990), with LOD scores greater than 3 being significant. The PRLR locus was closely linked to three markers which are mapped to pig chromosome 16 of the published PiGMap linkage map. A multiple point analysis was also done to produce a best chromosome 16 map (Figure 3) involving all linked markers.



**Example 2****PCR TEST for Prolactin Receptor Genetic Marker**

The PCR amplification test was optimized with the following parameters.

**Primers:**

- 5 forward primer 5'-CCCAAAACAGCAGGAGAACG-3' (SEQ ID NO:1)  
reverse primer 5'-GGCAAGTGGTTGAAAATGGA-3' (SEQ ID NO:2)

**PCR conditions:**

10	Cocktail Mix	25uL reaction
	10X PCR buffer (Promega)	2.5uL
	25mM MgCl <sub>2</sub> (Promega)	2.0uL
	10mM dNTP's (Boehringer Mannheim)	0.5uL
	20pmol/uL forward primer	0.5uL
15	20pmol/uL reverse primer	0.5uL
	dd Sterile H <sub>2</sub> O	17.5uL
	12.5ng/uL DNA	1.5uL
20	Taq Polymerase (Promega)	0.125uL

- The first six reagents should be mixed and 18.5uL of this pre-mix added to each reaction tube. Add the DNA next and then overlay with a drop of sterile mineral oil. Place the reaction tubes on the terminal cycler held at 25 80°C. Mix the Taq with the remaining cocktail and add 5uL to the reaction tubes, making sure to submerge the tip beneath the oil.

**Thermal Cycler Program:**

- |    |    |                                |
|----|----|--------------------------------|
| 30 | 1. | 93°C 3 minutes                 |
|    | 2. | 93°C 30 seconds                |
|    | 3. | 60°C 1 minute                  |
|    | 4. | 72°C 1 minute                  |
|    | 5. | Return to step 2 for 34 cycles |
| 35 | 6. | 72°C 3 minutes                 |
|    | 7. | 4°C hold                       |

5 uL of the PCR product plus 2 uL of 6X loading dye should be placed on a 1% agarose gel to check. Run at 120V for 30 minutes and stain with Ethidium Bromide.

#### 5 AluI Digestion:

<u>Digestion Mix (per 20uL PCR product)</u>		<u>Each</u>
10X NEBuffer 2 (New England Biolabs)		2.5uL
8U/uL AluI (New England Biolabs)		0.5uL
10 dd Sterile H <sub>2</sub> O		2.0uL

Mix the reagents and add 5uL to each tube. Incubate the samples at 37°C overnight.

#### 15 Gel Electrophoresis:

The fragments are separated by loading the digest product plus 5uL 6X loading dye on a 6% NuSieve (FMC) agarose gel at 120 volts for 3 hours at room temperature. Stain gels with ethidium bromide. The fragment sizes of the PCR-RFLP are approximately 124, 110, 79, 77, and 67 base pairs with the polymorphic site being located in the 110 base pair fragment. When the polymorphic cut site is present a 90 base pair fragment is produced. Thus a heterozygote will have bands at 124,110,90,79,77 and 67. While homozygotes will have bands at 124,90,79,77 and 67 and 124,110,79, and 67 respectively.

25 Figure 4 is a diagram of the fragments obtained from the PCR test.(A is the allele with no AluI site, B is the allele having the AluI site).

### Example 3

#### 30 Association Of Genotype With Litter Size

The PCR test was run as detailed in Example 2 on several sows from Pig Improvement Company, (PIC). The animals used were PIC line 19 sows which farrowed within a six month period and gilts which were born during this period that would be kept as breeding stock. Blood or tissue samples were collected and shipped to the laboratory where the DNA was extracted and used in the PRLR PCR test. Females had one to three records used in the analysis. Estimated Breeding Value for Total Number Born (BV TNB) was estimated

using a mixed linear model where each successive parity of the sow is treated as a repeated record. Only the first three parities of a sow were used. The model includes the covariate of age at farrowing nested within parity, fixed effects of parity, service type (natural or AI), farm-month farrowed, and random permanent environmental and animal effects. Current  $h^2$  is assumed as 0.10 and repeatability as 0.21. Average Number Born (AV NB) was calculated by taking the arithmetic mean of total parities (1-3) for each female. Genotype comparisons were made for (BV TNB) and (AV NB) by averaging individual BV TNB and AV NB for each genotype. The results are seen in Table 1.

Table 1. Means for a sample of L19 animals by PRLR genotype

<b>BB Genotype</b>		
(n=18)	<u>BV TNB</u>	<u>AV NB</u>
Mean	0.2036	10.32
Std Dev	0.3984	1.746193
Std Err	0.0939	0.411838
<b>AB Genotype</b>		
(n=75)	<u>BV TNB</u>	<u>AV NB</u>
Mean AB	0.1203	9.66
Std Dev AB	0.5317	2.77238
Std Err AB	0.0622	0.320136
<b>AA Genotype</b>		
(n=109)	<u>BV TNB</u>	<u>AV NB</u>
Mean AA	0.0755	9.75
Std Dev AA	0.5757	2.513279
Std Err AA	0.0551	0.243064

#### EXAMPLE 4

##### Summary of Analyses of Prolactin Receptor With a Large White Line, a Meishan Synthetic Line and a Landrace Line

5 A total of 2,714 litter records from 1,077 sows were included in the litter size analyses. Traits included total number born (TNB) and number born alive (NBA) from five different PIC lines. The five lines examined were of Large White (two different origins) and Landrace origin, as well as synthetic lines consisting of 3/4 Duroc, 1/4 Large White, and Large White/Meishan  
10 origin. The PRLR genotype was shown to explain a statistically significant variation in litter size in three of the lines tested. Two of the lines did not show any statistically significant effect ( $P > 0.1$ , results not shown). The least square means for TNB and NBA for each of the three statistically significant lines are summarized in Table 2.

15 DNA was extracted from blood and tail tissue. DNA was analyzed as described in Example 2 above.

Models contained fixed effects of: herd-season, service type, prolactin receptor, parity (1,2,3+) and the covariable: ESR (estrogen receptor) and random effect:  
20 sire

Interactions among herd, ESR and prolactin receptor were tested also for significance. Heritability for the litter traits was assumed as 0.10 and repeatability as 0.21. Allele substitution effects were estimated by substituting for PRLR genotype a covariate heterozygote which included the  
25 number of A alleles present (0, 1, or 2). Dominance effects were estimated as the deviation of the heterozygote mean from the average of the homozygous genotype means.

##### Main conclusions:

30 • Large White synthetic

- Indications of a dominance effect.

The sample consisted of 400 sows with 1197 litter records. AA animals have a 0.66 pig/litter advantage in Number Born Alive (NBA) over the other two genotypes ( $p < 0.05$ ). There are indications of a  
35 dominance effect with the B allele.

- Meishan Synthetic

- Significant dominance effect (over-dominance) -over all parities but mainly in first parity.

5 The sample consisted of 261 sows with 832 litter records. There is evidence of an additive effect for TNB ( $P < 0.05$ ) and NBA ( $P < 0.05$ ) and an overdominance effect for NBA in this line ( $< 0.01$ ).

- Landrace Synthetic

- Indications of an additive effect.

10 The sample consisted of 416 sows with 685 litter records. A greater than one pig per litter difference between the two homozygous genotypes was detected for both TNB ( $P < 0.08$ ) and NBA ( $P < 0.1$ ), with the A allele being favorable.

15 Effects on TNB showed the same trends as NBA for each of the populations. The results of Table 2 indicate that PRLR has a significant effect on litter size as measured by TNB and NBA in three commercial lines. It is apparent that the background genetics of each different line plays a part in the manner and the magnitude that the trait is affected. In addition, significant differences  
20 were not found for average Birth Weight in any of the lines tested. This is a potentially valuable observation as there is normally an inverse relationship between litter size and average birth weight. The prolactin receptor allele may therefore provide a method of increasing the birth weight of larger litters.

Table 2. Least square means for each PRLR genotype across all parities for TNB, NBA, and average birth weight (ABW) for three commercial lines of pigs.

Commercial Line	PRLR Genotype	TNB	NBA
Large White Synthetic	AA	12.51	12.39
	AB	12.35	11.73
	BB	12.71	11.73
			P<0.05
	Effects	a	-0.33 <sup>b</sup>
		d	-0.33 <sup>a</sup>
Meishan Synthetic	AA	13.64	12.94
	AB	14.35	13.74
	BB	13.96	13.27
		P<0.05	P<0.05
	Effects	a	0.16 <sup>b</sup>
		d	0.55 <sup>b</sup>
Landrace Synthetic	AA	12.13	11.33
	AB	11.72	10.92
	BB	10.98	10.31
		P<0.08	P<0.10
	Effects	a	0.51 <sup>b</sup>
		d	0.17

5 a=additive effect; d=dominance effect; effects are significant at <sup>a</sup>P<0.1, <sup>b</sup>P<0.05, <sup>c</sup>P<0.01

### EXAMPLE 5

10

#### Variation among different breeds

In addition, samples from seven breeds have been typed, including the U.S. breeds Chester White, Duroc, Hampshire, Landrace, and Yorkshire; the Chinese Meishan; and the European Large White (Table 3).

Table 3.

Breed	Genotype Frequencies			Allele Frequencies	
	AA	AB	BB	A	B
Landrace n=9	.56	.33	.11	.72	.28
Duroc n=10	.5	.5	0	.79	.21
Yorkshire n=12	0	.75	.25	.37	.63
Chester White n=10	.1	.3	.6	.25	.75
Hampshire n=11	0	.09	.91	.05	.95
Meishan n=9	.33	.44	.22	.56	.44
Large White n=11	.09	.46	.45	.32	.68

Some breed differences exist for gene frequencies at PRLR. The existence of a polymorphism located in the 3' region of the gene is interesting because PRLR alternative splicing is seen in this region of the gene in other species. The allele frequency differences among breeds suggest that one allele may have been selected for in some populations and against in others.

The following references are hereby incorporated in their entirety by reference:

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- 25 Rothschild, M., Jacobson, C., Vaske, D., Tuggle, C., Wang, L., Short, T., Eckardt, G., Sasaki, S., Vincent, A., McLaren, D., Southwood, O., van der Steen, H., Mileham, A., Plastow, G. (1996). The Estrogen Receptor Locus is Associated With a Major Gene Influencing Litter Size in Pigs. *Proc. Natl. Acad. Sci.* 93, 201-205.
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**What is claimed is:**

1. A method of screening pigs to determine those more likely to produce larger litters comprising: obtaining a sample of genetic material from a pig;  
5 and assaying for the presence of a polymorphism in the prolactin receptor gene in said sample which is associated with increased litter size.
2. The method of claim 1 wherein said step of assaying is selected from the group consisting of: restriction fragment length polymorphism (RFLP)  
10 analysis, heteroduplex analysis, single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE).
3. The method of claim 1 wherein said step of assaying for the presence of  
15 said polymorphism comprises the steps of: digesting said genetic material with a restriction enzyme that cleaves the pig prolactin receptor gene in at least one place; separating the fragments obtained from said digestion; detecting a restriction pattern generated by said fragments; and comparing said pattern with a second restriction pattern for the pig prolactin receptor  
20 gene obtained by using said restriction enzyme, wherein said second restriction pattern is associated with increased litter size.
4. The method of claim 3 wherein said restriction enzyme is AluI.
- 25 5. The method of claim 3 wherein said separation is by gel electrophoresis.
6. The method of claim 3 wherein said step of comparing said restriction patterns comprises identifying specific fragments by size and comparing the sizes of said fragments.

30

7. The method of claim 3 further comprising the step of amplifying the amount of pig prolactin receptor gene or a portion thereof which contains said polymorphism, prior to said digestion step.

5 8. The method of claim 3 wherein said polymorphism is a polymorphic AluI restriction site.

9. The method of claim 8 wherein said restriction site is located in the 3' coding region of the pig prolactin receptor gene.

10

10. The method of claim 7 wherein said amplification includes the steps of: selecting a forward and a reverse sequence primer capable of amplifying a region pig prolactin receptor gene which contains a polymorphic AluI site.

15 11. The method of claim 10 wherein said forward and reverse primers are selected from and based upon SEQ ID NO:3.

12. The method of claim 10 wherein said primers are SEQ ID NO:4 and SEQ ID NO:5.

20

13. The method of claim 12 wherein said forward primer is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4 and said reverse primer is selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:5.

25

14. The method of claim 12 wherein said primer set comprise SEQ ID NO:1 and SEQ ID NO:2.

15. The method of claim 6 wherein said step of detecting different sizes of  
30 said fragments comprises the steps of: separating said fragments by size using gel electrophoresis in the presence of a control DNA fragment of known size;

contacting said separated fragments with a probe that hybridizes with said fragments to form probe fragment complexes; and determining the size of separated fragments by detecting the presence of the probe fragment complexes and determining their relative positions with respect to said control DNA fragment.

16. The method of claim 15 wherein said restriction enzyme is AluI and said RFLP is extracted from the group consisting of a 110 base pair fragment and a 90 base pair fragment.

10

17. A method for identifying a genetic marker for pig litter size comprising the steps of: breeding male and female pigs of the same breed or breed cross or derived from similar genetic lineages; determining the number of offspring produced by each female pig; determining the polymorphism in the prolactin receptor gene of each female pig; and associating the number of offspring produced by each female pig with said polymorphism thereby identifying a polymorphism for pig litter size.

18. The method of claim 17 further comprising the step of selecting pigs for breeding which are predicted to have increased litter size by said marker.

19. The method of claim 17 wherein said analysis comprises digestion of PCR amplified DNA with the restriction enzyme AluI.

20. The method of claim 14 wherein said polymorphism associated with increased litter size is detected by use of first and second primers comprising at least 4 consecutive bases in SEQ ID NOS: 1 and 2.

21. A kit for evaluating a sample of pig DNA comprising, in a container, a reagent that identifies a polymorphism in the pig prolactin receptor gene.

22. The kit of claim 21 wherein said reagent is a primer that amplifies with the pig prolactin receptor gene or a fragment thereof.
23. The kit of claim 24 further comprising a DNA polymerase, a restriction enzyme which cleaves the pig prolactin receptor gene in at least one place; and first and second primers capable of amplifying a region of the pig prolactin receptor gene which contains a polymorphic site.
24. A primer for assaying for the presence of a polymorphic AluI site in the pig prolactin receptor gene wherein said primer comprises a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5.
25. A genetic marker associated with increased litter size in pigs, said marker comprising a polymorphism in the pig prolactin receptor gene.
26. The genetic marker of claim 25 wherein said polymorphism is a AluI restriction site.
27. The marker of claim 25 wherein said polymorphism is located in the 3' translated and nontranslated region of the pig prolactin receptor gene.
28. A DNA sequence from the pig prolactin receptor gene 3' translated and nontranslated region, said sequence consisting of SEQ ID NO:3.
29. A primer designed to amplify a polymorphic AluI restriction site in the pig prolactin receptor gene wherein said primer is 4 or more continuous bases from SEQ ID NO:3.

30. A primer designed to amplify a polymorphic AluI restriction site in the pig prolactin receptor gene where said primer is a reverse primer generated from the SEQ ID NO:3.

5 31. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises of the steps: determining the alleles of prolactin receptor present in a pig; determining the alleles of other markers for genes known to affect litter size; and selecting for animals with favorable combinations of alleles and  
10 against those carrying unfavorable combinations.

32. The method of claim 31 wherein a second gene determining litter size is ESR.

15 33. The method of claim 31 wherein the determination of prolactin receptor alleles comprises determining the presence of at least one allele associated with at least one DNA marker linked either directly or indirectly to prolactin receptor.

20 34. The method as claimed in claim 31 wherein the DNA marker is a microsatellite.

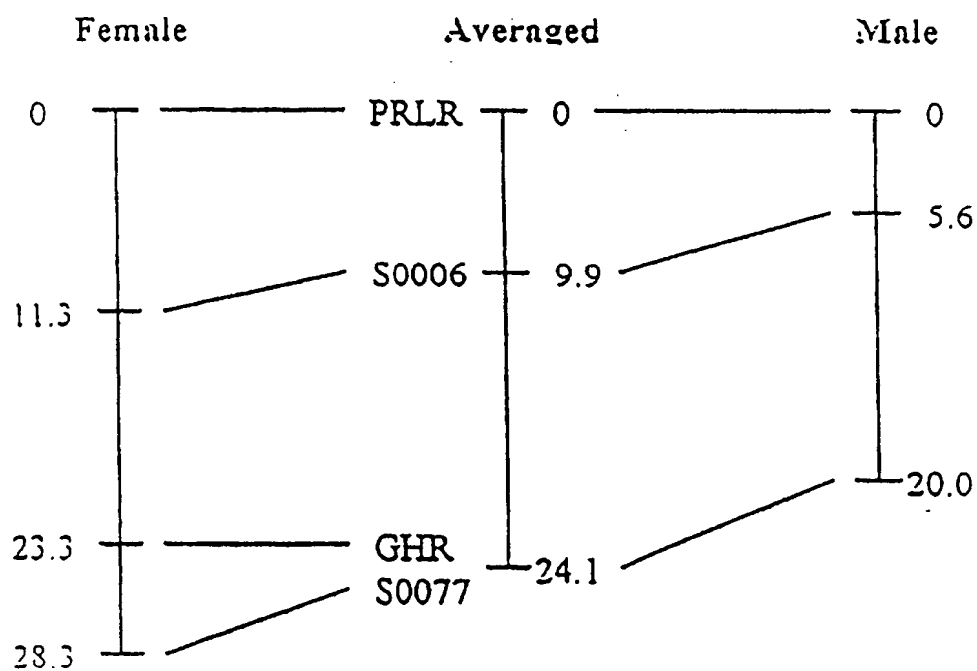
35. The method as claimed in claim 31 wherein the DNA marker is SW1305, S0077, S0006, SW2411, SW1035 and S0111.

25

36. The method as claimed in claim 31 wherein the DNA marker is growth hormone receptor (GHR).

30 37. The method of claim 1 wherein said marker is associated with maintenance of birth weight of the larger litters.

38. A method of screening pigs to determine those more likely to produce larger litters comprising: obtaining a sample of genetic material from a pig; and assaying for the presence of a polymorphism in the prolactin receptor gene in said sample which is associated with the maintenance of birth weight of
- 5 larger litters.



*Fig. 3*

AA

AB

BB

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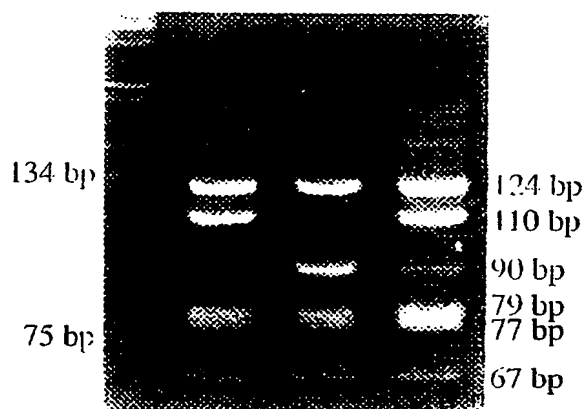
*Fig. 4*



AAGTCAACAA AGATGGAGCA CTGGCGTTGC TCCCAAAACA GCAGGAGAAC  
 GGCGACCGGC CGGAGAAGGC TGGCGCCCCT GAAACCAGCA AGGAATACG  
 CCCAGGTGTC CCGGGTGATG GATAACCACA TCCTGGTGTT AGTGCAGGAT  
 CCGCGAGCTC GAAACGTGGC TCCGTTTGAA GAACCAACCA AGGAGACCCC  
 GCCATCCCGG CCGCAGAATC CAGCTGCGAA AGACCTGGCC G/AGCTTCACCA  
 CGGCCCCGGG CCACTGCAGA CACCCGCTGG GTGGGCTGGA TTACCTCGAT  
 CCCGCAGGCT TTATGCACTC CTTTCAGTGA GAGCTTGGTT CATGGGATGA  
 TGGGTTACAA GGTGGGGTTT TTTTCAGGTC GCACTACGTG AAATGCACTC  
 TACCAGAGAA AGCTCGAAAA TGGGGTTAGA ATGACACTAC CCAGACTCAC  
 AATTCACTGC TTTTCTTCTT TTTTCTTCTT TTTTCTTCTT TTTTCTTCTT

G/A=G or A at polymorphic site

*Fig. 1*



*Fig. 2*



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/11508

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12Q 1/68; C07H 21/04

US CL : 536/24.33; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.51, 24.33; 435/6, 91.2, 91.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROTHSCHILD et al. The estrogen receptor locus is associated with a major gene influencing litter size in pigs. Proceedings of the National Academy of the Sciences, USA. January 1996, Vol. 93, pages 201-205, especially page 201.	1-38
A	US 5,374,526 A (ROTHSCHILD et al.) 20 December 1994 (20.12.94), see entire document.	1-38
A, P	WO 96/33288 A1 (DEKALB SWINE BREEDERS, INC. and NORTHERN ILLINOIS UNIVERSITY) 24 October 1996 (24.10.96), see entire document.	1-38
A, P	WO 96/41892 A1 (DALGETY PLC) 27 December 1996 (27.12.96), see entire document.	1-38



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

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## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Searched inventors and the keywords: prolactin receptor or prlr and genetic marker or gene or polymorphism and litter size or reproductive efficiency and sow or swine or boar or pig or porcine and alu PCR and growth hormone receptor in the databases APS, CAPLUS, SCISEARCH, LIFESCI, CABA, BIOSIS and WPIDS.